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DOI: https://doi.org/10.1111/j.1574-6968.2006.00500.x

Cloning and characterization of *Enterobacter sakazakii* pigment genes and *in situ* spectroscopic analysis of the pigment

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Received 19 August 2006; revised 2 October 2006; accepted 4 October 2006.
First published online 27 October 2006.

DOI:10.1111/j.1574-6968.2006.00500.x

Editor: Stephen Smith

Keywords
Enterobacter sakazakii; bacterial pigment; carotenoid biosynthesis genes; *in situ* visible microspectroscopy; *in situ* resonance Raman microspectroscopy.

Abstract

Enterobacter sakazakii is considered an opportunistic foodborne pathogen that is characterized by formation of yellow-pigmented colonies. Because of the lack of basic knowledge about *Enterobacter sakazakii* genetics, the BAC approach and the heterologous expression of the pigment in *Escherichia coli* were used to elucidate the molecular structure of the genes responsible for pigment production in *Enterobacter sakazakii* strain ES5. Sequencing and annotation of a 33.025 bp fragment revealed seven ORFs that could be assigned to the carotenoid biosynthesis pathway. The gene cluster had the organization *crt*E-*idi*-*XYIBZ*, with the *crtE*-*idi*-*XYIB* genes putatively transcribed as an operon and the *crt*Z gene transcribed in the opposite orientation. The carotenogenic nature of the pigment of *Enterobacter sakazakii* wt was ascertained by *in situ* analysis using visible microspectroscopy and resonance Raman microspectroscopy.

Introduction

Enterobacter sakazakii was first classified as 'yellow-pigmented *Enterobacter cloacae*' until it was recognized as a distinct species (Farmer *et al*., 1980). This was due to differences in biochemical reactions and after DNA–DNA hybridization comparison using *Enterobacter cloacae* and *Citrobacter divs*. (symptomatically similar to *Enterobacter sakazakii*), which showed only 50% association with either organism. The growth of yellow-pigmented colonies on trypticase soy agar (TSA) is currently used as, a criterion for identification of presumptive *Enterobacter sakazakii* isolates (http://www.cfsan.fda.gov/~comm/mmesakaz.html). However, the intensity of the pigmentation varies among strains, and nonpigmented variants are observed occasionally (Farmer *et al*., 1980).

Bacterial pigments have important functions in photosynthesis, and protection against photooxidative damage (Goodwin 1972; Olson 1993). The antioxidant activity of the pigment does not only play a role in the fitness of the organisms to persist in harmful environments but has also been reported to promote virulence in pathogenic bacteria (Reverchon *et al*., 2002; Liu *et al*., 2005; Clauditz *et al*., 2006). Both Liu *et al*. (2005) and Clauditz *et al*. (2006) have described a reduction in oxidant killing of xanthenoid-producing *Staphylococcus aureus* strains. This Gram-positive pathogen produces a yellow pigment at 37 °C. Reverchon *et al*. (2002) have described the relationship between indigoidine (a blue pigment) and pectate lyases, the main virulence determinant in the phytopathogen *Erwinia chrysanthemi*. Multiple gene clusters responsible for the synthesis of carotenoids have been identified in various carotenogenic bacteria including several *Enterobacteriaceae* species. Most of these gene clusters have the classic gene organization *crtEXYIBZ*. However, within a recent study rare gene clusters containing an additional gene *idi*, encoding isopentyl pyrophosphate isomerase, present between *crt*E and *crt*X genes, were reported in carotenoid gene clusters from environmental *Enterobacteriaceae* strains (Sedkova *et al*., 2005).

To date, nothing is known about pigment production in *Enterobacter sakazakii*; thus, it was the aim of this study to

elucidate the nature of and the molecular basis for this feature.

By using the BAC approach and the heterologous expression of the pigment in *Escherichia coli,* seven genes were identified that could be assigned to the carotenoid biosynthesis pathway. The organization of the gene cluster showed homology to the one from *Panotoea agglomerans (crtE-idi-XYIBZ*). The carotenogenic nature of the yellow pigment was confirmed by means of *in situ* visible microspectroscopy and *in situ* resonance Raman microspectroscopy.

Materials and methods

Bacterial strains and plasmid

Enterobacter sakazakii strain ES5, a clinical isolate (provided by Dr R. Zbinden, Department of Medical Microbiology, University of Zurich), TransforMax EPI 100 electro competent *Escherichia coli* (Epicentre) and the pIndigoBAC-5 (HindIII-Cloning Ready) vector (Epicentre) were used for the BAC cloning approach.

Construction of the BAC library and screening for pigmented clones

Construction of the BAC library was performed according to the method published by Lehner *et al.* (2006). The pigment expression of the *Escherichia coli* transformants was assessed visually on Luria–Bertani (LB) agar media containing 12.5 μg mL^{-1} chloramphenicol) after 48–96 days of incubation in a sterile hood under constant light at room temperature.

Sequencing and annotation

Sequencing of *Escherichia coli* transformant 9E10 insert DNA was outsourced (MWG, Ebersberg, Germany). Automated annotation of the assembled sequence was performed using the PEDANT (Frishman *et al.*, 2003) software system, and prediction of coding sequences was performed with ORPHEUS (Frishman *et al.*, 1998a, b). Translated coding sequences (CDSs) longer than 180 nucleotides were searched using BLAST (Altschul *et al.*, 1990) and FASTA (Pearson & Lipman 1988) against SWISSPROT/UNIPROT protein sequence database (cut-off *e*-value $\leq 1e-04$) and further analyzed by searching against various motif and domain libraries (see http://pedant.gsf.de/about.html for a detailed list of the methods and databanks used). The comprehensive data collected automatically for each CDS by PEDANT were subsequently used as a basis for careful manual annotation. CDSs were assigned to functional categories according to the functional role catalogue FunCat (Ruepp *et al.*, 2004). Proteins with amino acid sequence homology to characterized proteins (either by knock-out/complementation experiments, protein expression or by known 3D structure) were annotated as strongly similar to known proteins (> 40% amino acid sequence identity to characterized protein) or as similar to known proteins (> 20% amino acid sequence identity to characterized protein). CDSs with sequence homology to not yet functionally characterized proteins were classified as conserved hypothetical proteins (> 30% amino acid sequence identity to first blast hit) or hypothetical proteins (> 20% amino acid sequence identity to first blast hit). CDSs showing no significant homology to proteins in public databases were annotated as unknown protein.

Growth conditions for *in situ* spectroscopic analysis

Enterobacter sakazakii strain ES5 wt and *Escherichia coli* transformant 9E10 were grown on LB agar and LB medium containing 12.5 μg mL^{-1} chloramphenicol, respectively, for 48 h at room temperature in a sterile hood under constant light.

Visible microspectroscopy

Visible spectra were recorded in the transmission mode with a J & M TIDAS MSP 800 diode array spectrometer (J & M Analytische Mess- und Regeltechnik GmbH, Aalen, Germany) equipped with a ZEISS Axioplan 2 imaging microscope and using the J & M SPECTRALYS VERSION 1.82 software. Wavelength calibration was carried out using a holmium filter. A slice (5 × 5 mm) was cut out with a scalpel from the agar with the least possible growth medium and placed on the microscope slide without the cover-slip. The light was focused on the sample using a × 40 objective.

Raman microspectroscopy

Raman spectra were recorded at room temperature with a RENISHAW inVia Raman Microscope (Renishaw, Old Town, Gloucestershire, England) equipped with an integrated Leica DML microscope and using the RENISHAW WIRE 2.0 software. The excitation was provided by a LaserPhysics Ar^+ laser (514.5 nm) with an output power of 20 mW at the source. The spectrometer was calibrated using silicon. A part of the colony (5 × 5 mm) was placed on the microscope slide without the cover-slip. The laser beam was focused on the sample using a × 20 objective.

European molecular biology laboratory (EMBL) nucleotide sequence database accession number

The EMBL Nucleotide Sequence Database accession number for the BAC sequence reported in this paper is AM384990.

Results and discussion

Cloning of yellow pigment genes

The BAC library of *Enterobacter sakazakii* strain ES5 in *Escherichia coli* displayed colonies with a yellow phenotype in the ratio of 7 : 1100. *Escherichia coli* transformant 9E10 was selected randomly for sequencing and analysis of the insert DNA. An insert size of 33.025 bp was determined for the recombinant plasmid in this transformant. By automated sequence analysis 37 ORFs were predicted. Manual deletion of ORFs containing < 180 nucleic acids and/or without significant homology to proteins in public databases resulted in 28 translated CDSs with an average length of 954.4 bp and an average GC content of 60.5%. The fully annotated sequence of *Escherichia coli* transformant 9E10 was deposited in EMBL GenBank.

Organization of the carotenoid biosynthesis gene cluster in *Enterobacter sakazakii*

Automated annotation of the insert DNA sequence obtained from *Escherichia coli* transformant 9E10 revealed the presence of seven genes that could be assigned to the carotenoid pathway. The gene cluster had the organization *crtE-idi-XYIBZ*, homologous to a gene cluster originally reported for *Pantoea agglomerans* (GenBank accession number M87280). Considering the presence of a relatively large noncoding region downstream of the putative gene cluster, leaving space for a possible promotor and RBS and the small intergenic spaces between the adjacent ORFs, it can be hypothesized that the *crt*E-*idi*-XYIB genes are most likely transcribed as an operon and that the *crt*Z gene is transcribed in the opposite direction (Fig. 1). This would be in agreement with previous reports (Sedkova *et al.*, 2005).

These data suggest that the proposed pathway for synthesis of the pigment produced in *Enterobacter sakazakii*, starting from the C_{15} compound farnesyl pyrophosphate (FPP), which is present in both carotenogenic and noncarotenogenic bacteria, includes the extension of the FPP to common C_{40} carotenoids, such as β-carotene, by activity of geranylgeranyl pyrophosphate synthase (CrtE), phytoene synthase (CrtB), phytoene dehydrogenase (CrtI) and lycopene cyclase (CrtY). The enzymes β-carotene hydroxylases (CrtZ) and zeaxanthin glucosylases (CrtX) carry out subsequent modifications of β-carotene to generate a variety of C_{40} carotenoids. In addition, the carotenoid gene cluster present in *Enterobacter sakazakii* contained an *idi* gene, encoding isopentyl pyrophosphate isomerase. According to the study by Sedkova *et al.*(2005), the *idi* gene is responsible for increased carotenoid titers.

The comparison of amino acid identities for the carotenoid synthesis genes from *Enterobacter sakazakii* and different *Enterobacteriaceae* environmental and type strains is summarized in Table 1.

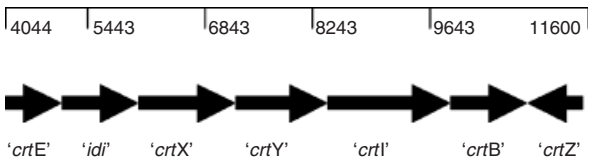

Fig. 1. Putative organization of the carotenoid synthesis gene cluster in *Enterobacter sakazakii* strain ES5 (scale: nucleotide position on AM384990).

Table 1. Comparison of amino acid identities for the carotenoid synthesis genes from *Enterobacter sakazakii* and different *Enterobacteriaceae* environmental and type strains

Nucleotide position on AM384990	Gene	% AA identity/Organism/Accession number*
4044-4949	*crtE*	71%/ *Enterobacter* bacterium DC404/ Q2VJ80_9ENTR
		67%/ *Enterobacter* bacterium DC413/ Q2VJ68_9ENTR
		66%/ *Pantoea ananas*/P21684
4992-6035	*idi*	72%/ *Enterobacter* bacterium DC404/ Q2VJ79_9ENTR
		69%/ *Enterobacter* bacterium DC413/ Q2VJ67_9ENTR
		68%/ *Enterobacter agglomerans*/IDI2_ENTAG
6032-7306	*crtX*	60%/ *Enterobacter* bacterium DC413/ Q2VJ66_9ENTR
		58%/ *Enterobacter* bacterium DC416/ Q2VJ73_9ENTR
		57%/ *Pantoea agglomerans*/CRTX_PANAN
7303-8478	*crtY*	65%/ *Enterobacter* bacterium DC404/ Q2VJ78_9ENTR
		63%/ *Enterobacter agglomerans*/CRTY_ENTAG
		58%/ *Pantoea ananas*/CRTY_PANAN
8471-9949	*crtI*	82%/ *Pantoea ananas*/CRTI_PANAN
		82%/ *Enterobacter agglomerans*/ Q206M4_ENTAG
		79%/ *Enterobacter* bacterium DC404/ Q2VJ77_9ENTR
9946-10917	*crtB*	74%/ *Enterobacter* bacterium DC413/ Q2VJ63_9ENTR
		73%/ *Enterobacter* bacterium DC404/ Q2VJ76_9ENTR
		71%/ *Enterobacter* bacterium DC260/ Q2VJ82_9ENTR
10805-11347	*crtZ*	77%/ *Pantoea ananas*/CRTZ_PANAN
		76%/ *Escherichia vulneris*/CRTZ_ESCVU
		62%/ *Paracoccus sp.*/CRTZ_PARS1

*SWISSPROT/UNIPROT accession numbers.

In situ spectroscopic analysis of the *Enterobacter sakazakii* pigment

In order to obtain information on the nature of the pigment present in *Enterobacter sakazakii*, *in situ* visible spectroscopy was performed on colony material of the wt ES5 strain as well as one of the yellow-pigmented *Escherichia coli* transformants. In Figure 2, the visible spectra of the *Enterobacter sakazakii* wt, the nonpigment producing *Escherichia coli* EPI 100 and the *Escherichia coli* transformant 9E10 are shown. The spectra for *Enterobacter sakazakii* ES5 and *Escherichia coli* transformant 9E10 had absorption peaks at 432, 458 and 485 nm, whereas no such peaks were detected for *Escherichia coli* EPI 100. A spectrum showing absorption bands between 400 and 500 nm with three maxima, the middle band having the highest intensity, is characteristic for a carotenoid (Britton *et al.*, 2004).

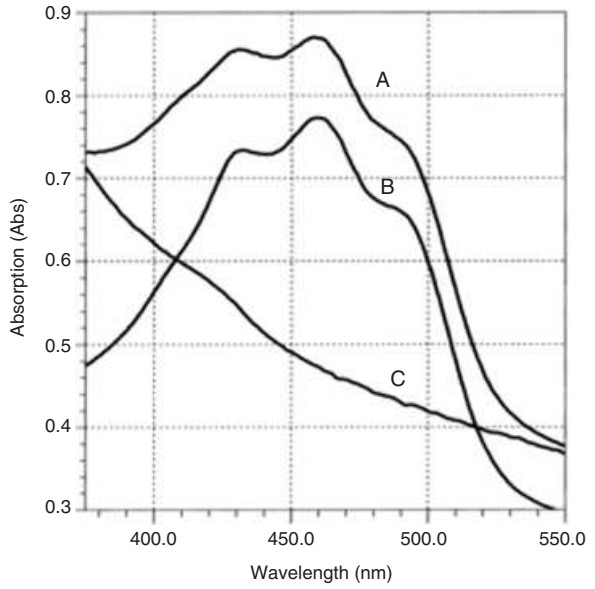

Fig. 2. Visible spectra of colony material of *Enterobacter sakazakii* ES5 wt (a), *Escherichia coli* transformant 9E10 (b) and *Escherichia coli* EPI 100 (c).

The *in situ* Raman spectrum of the *Enterobacter sakazakii* ES5 wt (Figure 3) displays strong peaks at 1521 cm^{-1} (ν_1) and at 1157 cm^{-1} (ν_2) as well as a peak of medium intensity at 1005 cm^{-1}. The strong bands within the 1500–1550 cm^{-1} and 1150–1170 cm^{-1} ranges are attributed to in-phase C = C (ν_1) and C–C stretching (ν_2) vibrations of the polyene chain, respectively, whereas the peak of medium intensity in the 1000–1020 cm^{-1} region is attributed to in-plane rocking modes of CH_3 groups attached to the polyene chain (Schulz *et al.*, 2005). The wavenumber positions of the ν_1 and ν_2 modes in the Raman spectrum are in very good agreement with published data (Merlin 1985; Schulz *et al.*, 2005) for carotenoids in biological matrices with nine conjugated carbon–carbon double bonds in the main chain. In this context, it is noteworthy to mention that the band position of ν_1 is dependent on the 'effective conjugated chain length' (Withnall *et al.*, 2003) and not on the number of conjugated double bonds.

Taking into account, on the one hand, the nine conjugated double bonds in the main chain and, on the other, the shape of the visible spectrum (Britton *et al.*, 2004), the following common bacterial carotenoids can be considered: β-carotene and the xanthophylls β-cryptoxanthin (β-carotene monool), zeaxanthin (β-carotene diol) as well as possible glycosides of the aforementioned xanthophylls. β-carotene, β-cryptoxanthin and zeaxanthin were identified in environmental *Enterobacteriaceae* strains by Sedkova *et al.* (2005).

Within this study, the nature of the yellow pigment produced in *Enterobacter sakazakii* was elucidated by both molecular and chemical/analytical means. However, there is still a lack of knowledge about the regulation of the expression of this feature. Initial experiments showed that the pigment production is subject to catabolite repression in

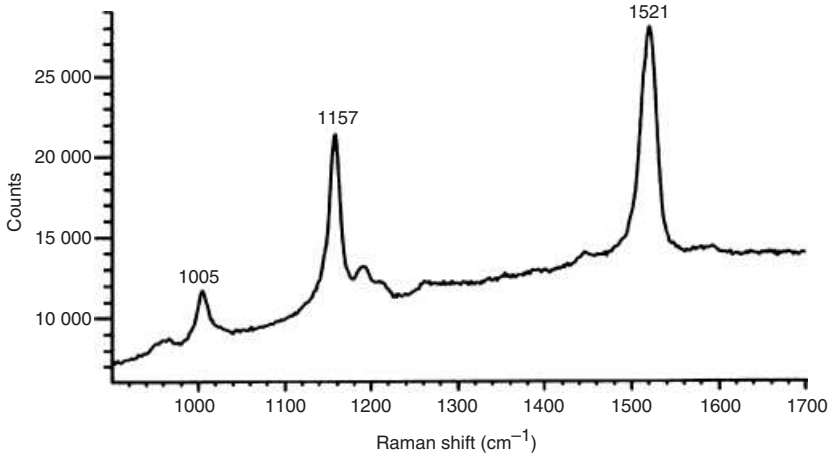

Fig. 3. Raman spectrum of *Enterobacter sakazakii* ES5.

yellow *Escherichia coli* transformants but not in *Enterobacter sakazakii* strain ES5 wt, thus suggesting alternative regulatory controls for the expression of this feature in *Enterobacter sakazakii* wt organisms. Analysis of the sequence information available from *Escherichia coli* transformant 9E10 suggests the presence of at least two regulatory structures (ribosomal polymerase D sigma 70 factor and putative transcriptional regulator) that could serve as target for further investigations on the regulatory mechanisms involved in the *Enterobacter sakazakii* pigment gene expression.

Moreover, these data are the basis for further research in *Enterobacter sakazakii* into the influence of pigmentation on environmental persistence as well as on virulence.

Acknowledgements

The authors thank Ms Sonja Ketterer (Forensic Scientific Laboratory, State Police, CH-9001 St Gallen, Switzerland) for technical assistance with visible microspectroscopy and Raman microspectroscopy.

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